Three-Photon Induced Fluorescence of the Calcium Probe Indo-1

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ABSTRACT   We report the calcium-dependent emission spectral properties of the calcium probe Indo-1 for three-photon excitation. We found that Indo-1 could be readily excited with the femtosecond pulses from a mode-locked Ti:sapphire laser at 885 nm. This wavelength is too long for two-photon excitation, which is expected to occur for wavelengths no longer than twice the longest single-photon absorption wavelength of 400 nm. For excitation at 885 nm the emission intensity was found to depend on the cube of the laser power, as expected for simultaneous interaction with three photons. At wavelengths below 840 nm the emission intensity depends on the square of the laser power, indicating two-photon excitation at shorter wavelengths. The intensity decays of Indo-1 were found to be dependent on Ca^{2+} and essentially identical for one- and three-photon excitation. The emission anisotropy of Indo-1 was found to be considerably higher for three-photon excitation than for one-photon excitation, consistent with $\cos^6\theta$ photoselection, as compared with $\cos^2\theta$ photoselection for one-photon excitation. The high values of the anisotropy are in agreement with those expected for a three-photon process. Calcium-dependent emission spectra were observed for Indo-1 with three-photon excitation, demonstrating that three-photon excitation of Indo-1 can be used for calcium imaging by emission intensity ratio measurements. The calcium-dependent emission spectra indicate a higher three-photon cross-section for the calcium-free form of Indo-1 than for the calcium-bound form. The possible advantages of three-photon excitation include the availability of the appropriate wavelengths with solid-state lasers, enhanced spatial resolution due to a reduced size of the excited volume, absence of light quenching, and possibly high selectivity of the three-photon excitation process.

INTRODUCTION

During the past several years, there has been an increased use of intense laser pulses as an excitation source for time-resolved fluorescence spectroscopy and for fluorescence imaging microscopy. Although these laser sources are most often used for one-photon excitation, the high peak power of picosecond and femtosecond laser pulses can result in two-photon excitation, wherein the fluorophore simultaneously absorbs two long-wavelength photons to yield the first excited singlet state. Initially, two-photon spectroscopy was used as a tool to study the excited-state symmetry of organic chromophores (Friedrich and McClain, 1980; Wirth et al., 1981; Birge, 1983). More recently two-photon excitation has been observed for aromatic amino acids and proteins (Rehms and Callis, 1993; Sammeth et al., 1990; Lakowicz et al., 1992a, 1994b; Lakowicz and Gryczynski, 1993), for membrane-bound fluorophores (Lakowicz et al., 1992c), and for fluorophores bound to DNA (Lakowicz and Gryczyński, 1992; Gryczynski and Lakowicz, 1994). Two-photon excitation has also been applied to fluorescence microscopy, where it provides intrinsic "confocal" excitation only in the focal region of the sample (Denk et al., 1990; Webb, 1990; Hell et al., 1994; Stelzer et al., 1994)

and possibly improved rejection of background emission (Piston et al., 1992). Two-photon excitation has also been used to provide localized release of neurotransmitters and to localize ion channels in neuronal tissues (Denk, 1994; Denk et al., 1994). Although not directly connected with two-photon spectroscopy, the effects of intense excitation pulses have also been considered for one-photon excitation (Ansari and Szabo, 1993) and polarized fluorescence photobleaching (Hellen and Burghardt, 1994).

In the present report we describe the unexpected observation of three-photon-induced fluorescence of the calcium probe Indo-1. This probe is widely used for intracellular calcium imaging. Indo-1 is an emission-wavelength ratiometric probe (Grynkiewicz et al., 1985; Lückhoff, 1986; Owen et al., 1991; Popov et al., 1988; Gunter et al., 1988; Tsien, 1988) and is thus most often used in laser scanning confocal microscopy in which the excitation source is a focused laser beam at a single wavelength (Diliberto et al., 1994; Brelje et al., 1993). Three-photon excitation has only been observed infrequently (Pradere et al., 1966; Singh and Bradley, 1964; Cable and Albrecht, 1986), and to date there have been no studies of biochemical fluorophores with three-photon excitation, no time-resolved studies, and no reports of potential application of this phenomenon to biophysics.

In a previous study we found that Indo-1 displays a relatively high cross section for two-photon excitation at 702 nm (Szmacinski et al., 1993). We now show that Indo-1 can be excited by a still longer wavelength of 885 nm and that emission is due to three-photon excitation. Given the availability of these long wavelengths from Ti:sapphire lasers, three-photon excitation may have

widespread applications for excitation of UV-absorbing fluorophores.

THEORY

Frequency-domain fluorometry

The intensity decays of Indo-1 were measured using the frequency-domain method (Lakowicz et al., 1984; Gratton et al., 1984). The intensity decay was assumed to be multi-exponential:

$$I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i}, \qquad (1)$$

where α_i are the preexponential factors, τ_i are the decay times, and n is the number of exponential components. The fractional intensity of each component in the steady state emission is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \qquad (2)$$

In frequency-domain fluorometry, the sample is excited with an intensity-modulated light source, in the present case the output of a mode-locked Ti:sapphire laser or of a cavity-dumped dye laser. The phase angle (θ_ω) and the modulation (m_ω) of the emission are related to the intensity decay parameters, α_i and τ_i, and modulation frequency ω by

$$\phi_\omega = \arctan(N_\omega/D_\omega), \qquad m_\omega = (N_\omega^2 + D_\omega^2)^{1/2}. \qquad (4)$$

where

$$N_\omega = \frac{1}{J} \sum_{i=1}^{n} \frac{\omega \alpha_i \tau_i^2}{1 + \omega^2 \tau_i^2}, \quad D_\omega = \frac{1}{J} \sum_{i=1}^{n} \frac{\alpha_i \tau_i}{1 + \omega^2 \tau_i^2},$$

$$\qquad (5)$$

$$J = \sum_{i=1}^{n} \alpha_i \tau_i.$$

The values α_i and τ_i are determined by minimization of the goodness-of-fit parameter

$$\chi_R^2 = \frac{1}{\nu} \sum_{\omega,k} \left(\frac{\phi_\omega - \phi_{\omega c}}{\delta \phi}\right)^2 + \frac{1}{\nu} \sum_{\omega} \left(\frac{m_\omega - m_{\omega c}}{\delta m}\right)^2 \qquad (6)$$

where the subscript c indicates calculated values for known values of α_i and τ_i, $\delta\phi$ and δm are the experimental uncertainties in the measured phase and modulation values, and ν is the number of degrees of freedom.

For the present paper we used the single correlation time anisotropy decay model

$$r(t) = r_{0i} e^{-t/\theta_r} \qquad (7)$$

where θ_r is the rotational correlation time, r_{0i} is the time 0 anisotropy, and the subscript i indicates the number of simultaneously absorbed photons. The values of r_0 and θ_r were recovered from least-squares analysis of the differential polarized phase angles and modulated anisotropies (Lakowicz et al., 1993), using an expression similar to Eq. 6. For the global analysis the same correlation time was used for each excitation wavelength (k), the r_{0i} values were distinct for each wavelength, and the sum in Eq. 6 was extended over the excitation wavelengths.

Fluorescence anisotropy with multi-photon excitation

The fluorescence anisotropy is a measure of the displacement of the emission transition moment from the direction of the polarized excitation. For vertically polarized (z axis) excitation with one or more photons the anisotropy is given by (Weber, 1966; Kawski et al., 1993)

$$r_0(\theta, \beta) = \left(\frac{3}{2}\langle\cos^2\theta\rangle - \frac{1}{2}\right)\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \qquad (8)$$

where θ is the angle from the z axis, and β is the angle between the absorption and emission transition moments.

The subscript 0 indicates the absence of rotational diffusion during the excited state lifetime or the time 0 anisotropy. The average value of $\cos^2\theta$ depends upon the type of photoselection. The value of $\langle\cos^2\theta\rangle$ is given by

$$\langle\cos^2\theta\rangle = \frac{\int_0^{\pi/2} \cos^2\theta f_i(\theta)\, d\theta}{\int_0^{\pi/2} f_i(\theta)\, d\theta} \qquad (9)$$

when $f_i(\theta)$ is the directional distribution of the excited state (Lakowicz et al., 1992b). For one-photon excitation this distribution is given by

$$f_1(\theta) = \cos^2\theta \sin\theta \qquad (10)$$

For one-photon excitation Eq. 7 becomes

$$r_{0i}(\beta) = \frac{2}{5}\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right). \qquad (11)$$

The factor of 2/5 originates with $\cos^2\theta$ photoselection (Eq. 10). For colinear transitions ($\beta = 0$) the fundamental anisotropy (r_{01}) without rotational diffusion is 0.40. For nonzero values of β the anisotropy ranges from 0.40 to -0.20.

The anisotropy expected for two- (r_{02}) or three- (r_{03}) photon excitation can be calculated using

$$f_2(\theta) = \cos^4\theta \sin\theta \qquad (12)$$

$$f_3(\theta) = \cos^6\theta \sin\theta \qquad (13)$$

where the subscript (2 or 3) refers to two- or three-photon excitation. Substitution into Eq. 7 yields

$$r_{02}(\beta) = \frac{4}{7}\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \tag{14}$$

$$r_{03}(\beta) = \frac{2}{3}\left(\frac{3}{2}\cos^2\beta - \frac{1}{2}\right) \tag{15}$$

For two- and three-photon excitation the maximal anisotropies for $\beta = 0$ are 0.571 and 0.667, respectively. Hence, for colinear transitions three-photon excitation is expected to result in a more highly oriented excited-state population. Observation of a larger anisotropy for three- versus two-photon excitation provides strong evidence for three-photon excitation. The anisotropy values for nonzero values of β are summarized elsewhere (Kawski et al., 1993).

We note that the above description is somewhat simplified and that multi-photon transitions are more correctly described as tensors (Wan and Johnson, 1994a,b; Callis, 1993; Chen and Van Der Meer, 1993). Under special conditions the anisotropy for two-photon excitation can be as high as 0.61 (Callis, 1993). For the present data the simple theory described in Eqs. 8–15 is adequate for interpretation of the results.

In the presence of rotational diffusion the steady-state anisotropy (r_i) is lower than the time 0 anisotropy. The steady-state anisotropy is related to the value of r_{0i} and to the rotational correlation time θ_r by

$$\frac{r_{0i}}{r_i} = 1 + \frac{\tau}{\theta_r} \tag{16}$$

where τ is the mean decay time. The mean decay time can be calculated using

$$\tau = \sum_i f_i \, \tau_i. \tag{17}$$

EXPERIMENTAL METHODS

Indo-1 was obtained from Molecular Probes and used without further purification. Ca^{2+} concentrations were obtained using the Calibrated Calcium Kit II, also from Molecular Probes, containing 10 mM CaEGTA and EGTA, 100 mM KCl, and 10 mM 3-(N-morpholino)propanesulfonic acid at pH 7.2. Calcium concentrations above 40 μM were obtained by adding $CaCl_2$ to the 40 μM calcium calibration solution. Frequency-domain intensity and anisotropy decays with three-photon excitation were obtained on instrumentation described previously (Lakowicz and Maliwal, 1985; Laczko et al., 1990). For one-photon excitation we used the frequency-doubled and cavity-dumped output of a rhodamine 6G dye laser at 295 nm, with a repetition rate of 3.796 MHz, or the frequency-doubled output of a pyridine 1 dye laser at 351 nm. The pulse width was near 7 ps. Three-photon excitation was provided by a femtosecond mode-locked Tsunami Ti-sapphire laser from Spectra Physics.

The repetition rate of 80 MHz was held fixed by the Loc-to-Clock accessory. The repetition rate was divided by 8 by the Loc-to-Clock electronics, and used as the 10-MHz reference signal for the frequency domain instrument. The pulse width was near 80 fs.

The fundamental output of the Ti:sapphire (840–900 nm) was brought directly to sample compartment and focused with a laser-quality lens (2 cm focal length). The emission was isolated with three Corning 4-96 filters for intensity measurements. The samples were stirred during the measurements, which was necessary to obtain a stable signal. For intensity decay measurements on the long wavelength side of the Indo-1 emission we also used a Corning 3-72 emission filter, in addition to the Corning 4-96 filters. Differences in the decay time of the calcium-free and calcium-bound forms of Indo-1 are most apparent at long emission wavelengths (Szmacinski et al., 1993). For anisotropy decay measurements, and the related intensity decay measurements, the entire emission of Indo-1 was observed through the 4-96 filters, which transmit from 350 to 600 nm. Intensity and intensity-decay measurements were performed using magic angle conditions. For emission spectra we used a SLM 8000 spectrofluorometer with a 10-nm bandpass. Solutions were in equilibrium with the air. The signals from the solvents alone were less than 0.1% of that observed in the absence of Indo-1. For measurements of the dependence of the emission on laser intensity the peak power was attenuated with neutral density filters. To avoid any effects of widening the laser pulses by the neutral density filters, a single filter of the same design and thickness, but varying optical density, was used for the intensity measurements at various peak powers. All measurements were performed at room temperature near 20°C.

RESULTS

Emission spectra and anisotropies of Indo-1

Absorption and emission spectra of Indo-1 are shown in Fig. 1. The one-photon absorption spectra are seen to be dependent on Ca^{2+}, with the Ca^{2+}-bound form displaying a shorter wavelength absorption (Fig. 1, top). The emission spectra are also dependent on Ca^{2+}, with the Ca^{2+}-bound form of Indo-1 displaying a blueshifted emission (Fig. 1, bottom). As will be explained below, the concentration of Ca^{2+} was increased to 250 μM to ensure emission from only the Ca^{2+}-bound form of Indo-1 for excitation at 885 nm. Emission spectra of Indo-1 for excitation at 885 nm appear to be slightly shifted toward shorter wavelengths (about 5 nm at maxima) compared to the excitation at 295 nm. These spectra were collected using 10 m of 0.6-mm diameter optical fiber and an SLM 8000 spectrofluorometer. For excitation at 295 nm samples were diluted 10-fold and taken from the same sample compartment as for excitation at 885 nm. It should be noted that the signals for one-photon excitation were about 50-fold stronger than for three-photon excitation. Therefore, the minor differences between spectra
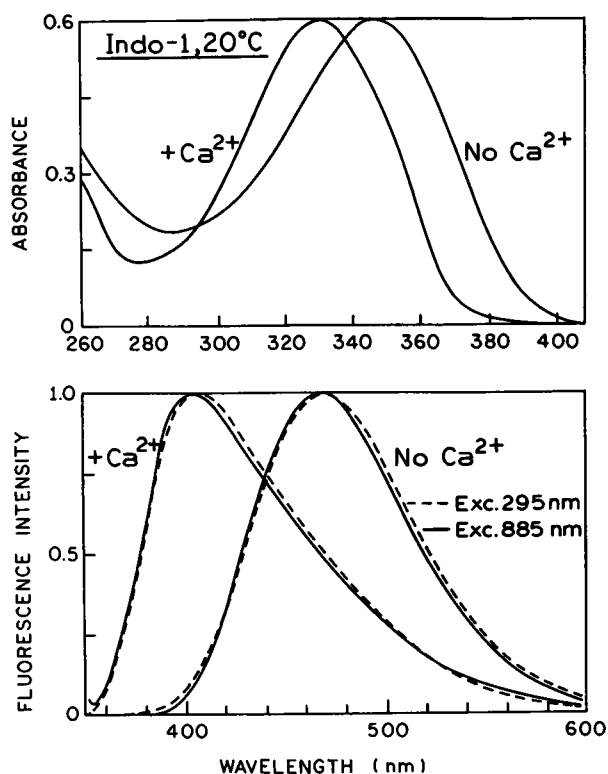
FIGURE 1 Absorption spectra of Indo-1 (*top*) and emission spectra (*bottom*) for one-photon (295 nm, – – –) and three-photon (885 nm, ——) excitation. For the emission spectra the concentrations of Indo-1 were 1.5 and 15 μM, for 295 and 885 nm excitation, respectively. For the calcium-bound form of Indo-1 [Ca^{2+}] = 40 μM for 295 nm and 250 μM for 885 nm excitation.

FIGURE 2 Dependence of the emission intensity of Indo-1 on laser power. The laser power is in milliwatts. [Indo-1] = 15 μM.

for one- and three-photon excitation are likely to within an experimental error, so that the emission spectra appear to be essentially identical for 295 and 895 nm excitation.

We were surprised by the observation of Indo-1 emission at this long excitation wavelength. Indo-1 is known to display a significant cross section for two-photon excitation (Szmacinski et al., 1993). However, two-photon excitation of Indo-1 at 885 nm seems unlikely because the one-photon absorption spectra do not display significant absorbance above 400 nm (Fig. 1, *top*), and thus one does not expect two-photon excitation at 885 nm. To determine the nature of the long-wavelength excitation we examined the dependence of the emission intensity on the incident laser power. These data show that the observed intensities of the calcium-free and calcium-bound forms of Indo-1 are proportional to the cube of the laser power (Fig. 2). In such experiments, with presumed three-photon excitation, it is important to consider the generation of the third harmonic by optical components or even the solution itself (Cable and Albrecht, 1986). Under the conditions of these experiments with 885-nm excitation, we could detect no signal at 295 nm, which indicates that the Ti:sapphire pulses were not being tripled by the solution or the optical components.

Three-photon excitation of Indo-1 is only observed for long excitation wavelengths. The intensity of Indo-1 be-
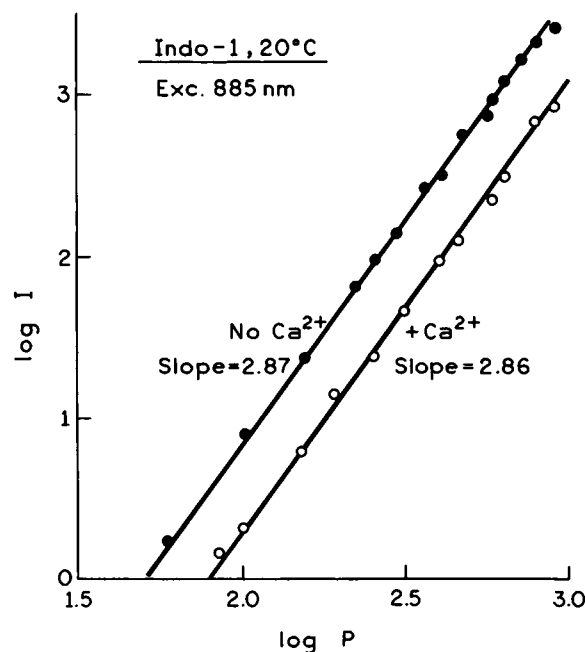
comes dependent on the square of the laser power for wavelengths below 840 nm (not shown). Addition of glycerol to the aqueous buffer seemed to increase the amount of two-photon versus three-photon excitation of Indo-1. The glycerol-induced red shift of absorption is only about 5 nm, and further studies are required to clarify the effects of glycerol on the multi-photon excitation.

To further characterize the emission of Indo-1 with 885-nm excitation we measured the intensity decays (Fig. 3) and anisotropy decays (Fig. 4), in the absence and presence of Ca^{2+}. The number of modulation frequencies was limited by the 80 MHz output of the Ti:sapphire laser, allowing measurements at only integer multiples of 80 MHz (Berndt et al., 1982; Alcala et al., 1986). The phase angles and modulations of Indo-1 in the presence and absence of Ca^{2+} were measured using the reference fluorophore 4-dimethyl-amino-4'-cyanostilbene (DCS) in methanol with a lifetime of 0.46 ns (Gryczynski et al., 1994). At 885 nm DCS displayed two-photon excitation, and the lifetime of DCS is independent of one- or two-photon excitation. DCS was thus a convenient standard for intensity decay measurement of Indo-1, because the same set of emission filters could be used for the sample and reference.

The multi-exponential analyses of the frequency-domain intensity decays of Indo-1 are summarized in Table 1. When the entire emission is observed the decays are only slightly dependent on Ca^{2+}. Essentially, the same multi-exponential intensity decay was observed for one- (295 nm) and three-photon (885 nm) excitation, suggesting that the same emitting state is observed independent of the mode of excitation.

To facilitate the potential use of Indo-1 as a lifetime-based calcium probe we measured the phase and modulation
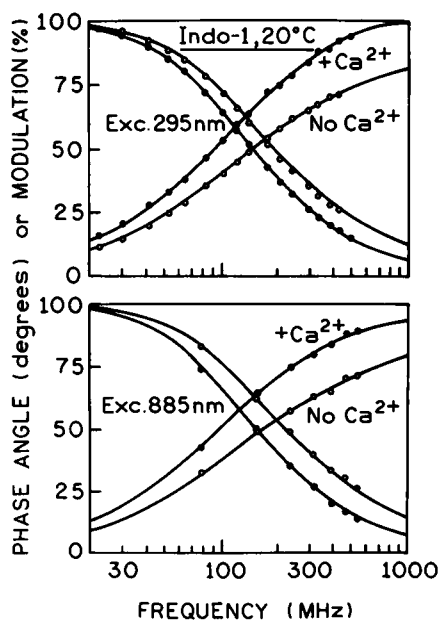
FIGURE 3 Frequency-domain intensity decay measurements of Indo-1 for one- and three-photon excitation at 295 and 885 nm, respectively. The emission was observed through three Corning 4-96 filters and one Corning 3-72 filter.

of the long-wavelength part of the emission spectra (Fig. 3). Long-wavelength observation was chosen because the intensity decay in this region of the emission spectrum is Ca^{2+}-dependent (Szmacinski et al., 1993). The intensity decay analysis is summarized in Table 2. The double-
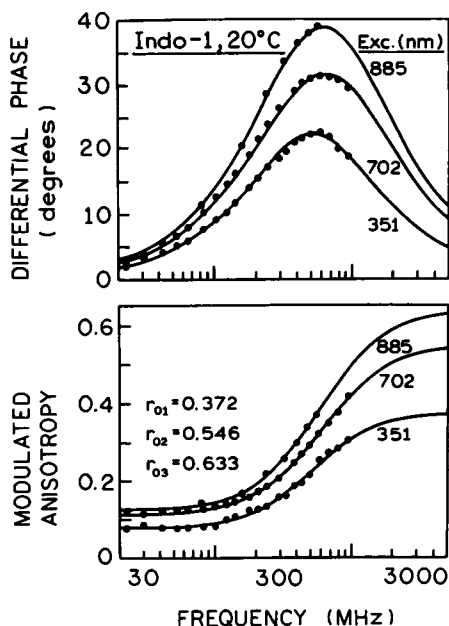

FIGURE 4 Frequency-domain anisotropy decay measurements of Indo-1 for one-, two-, and three-photon excitation at 351, 702, and 885 nm, respectively, in the absence of Ca^{2+} (Table 3). The emission was observed through three 4-96 filters.

TABLE 1 Intensity decay analysis of Indo-1 in the presence and absence of calcium for one- and three-photon excitation*

Excitation and [Ca^{2+}]	τ_i (ns)	α_i	f_i	$\chi_R^{2\ddagger}$
No Ca^{2+}				
295 nm, no Ca^{2+}	1.20	1	1	63.4
	0.18	0.295	0.051	
	1.38	0.705	0.949	2.3
885 nm, no Ca^{2+}	1.37	1	1	52.2
	0.20	0.201	0.032	
	1.56	0.799	0.968	3.9
+ Ca^{2+}				
295 nm, 40 μM Ca^{2+}	1.62	1	1	23.6
	0.28	0.123	0.022	
	1.71	0.877	0.978	2.1
885 nm, 250 μM Ca^{2+}	1.56	1	1	30.2
	0.23	0.143	0.022	
	1.71	0.857	0.978	4.4

*Observed through three 4-96 Corning filters, 350-600 nm, 20°C.
‡The uncertainties in Eq. 6 were $\delta\phi$ = 0.3 degrees for phase angles and δm = 0.007 for modulations (Lakowicz et al., 1993).

TABLE 2 Intensity decay analysis of Indo-1 in the presence and absence of calcium for one- and three-photon excitation*

Excitation and [Ca^{2+}]	τ_i (ns)	α_i	f_i	$\chi_R^{2\ddagger}$
No Ca^{2+}				
295 nm, no Ca^{2+}	1.33	1	1	30.9
	0.63	0.343	0.168	
	1.61	0.657	0.832	3.6
885 nm, no Ca^{2+}	1.08	1	1	52.0
	0.43	0.367	0.150	
	1.40	0.633	0.850	3.8
40 μM Ca^{2+}				
295 nm, 40 μM Ca^{2+}	2.18	1	1	241.5
	0.28	−0.313	−0.067	
	1.81	0.687	0.933	2.4
885 nm, 250 μM Ca^{2+}	2.02	1	1	76.8
	0.22	−0.224	−0.035	
	1.76	0.776	0.965	2.9

*Observed for long wavelength part of the emission spectra using 3-72 and 4-96 Corning filters, 445-600 nm, 20°C.
‡The uncertainties in Eq. 6 were $\delta\phi$ = 0.3 degrees for phase and δm = 0.007 for modulation (Lakowicz et al., 1993).

exponential model (Eq. 1) was sufficient to describe the intensity decays of Indo-1 in the presence and absence of Ca^{2+}. There is good agreement between intensity decay parameters, obtained for one- and three-photon excitation. The presence of negative amplitude for the Ca^{2+}-bound form has been observed earlier and discussed in terms of excited-state reaction (Szmacinski et al., 1993). The similarity of the Ca^{2+}-dependant intensity decays of Indo-1 for one- and three-photon excitation suggests that Indo-1 can be used for lifetime imaging with three-photon excitation.

We also examined the anisotropy decays of Indo-1 for one-, two-, and three-photon excitation at 351, 702, and 885 nm, respectively. We used 351 nm for one-photon excitation because the transition moments are nearly colinear at this wavelength, that is, r_{01} is near 0.4, whereas the value of

r_{01} is lower for 295-nm excitation. The lower value of r_{01} for 295-nm excitation indicates an overlap of electronic states for this excitation wavelength. The anisotropy decays of Indo-1 in the absence of Ca^{2+} for excitation at 351, 702, and 885 nm are shown in Fig. 4. It is interesting to notice that the differential polarized phase angles and modulated anisotropies are progressively larger for one-, two-, and three-photon excitation. These data can be used to recover the time 0 anisotropy using Eq. 7. The recovered time 0 anisotropy for three-photon excitation (r_{03}) was found to be 0.633, in contrast to a value of r_{01} = 0.372 for one-photon excitation at 351 nm (Table 3). The value of r_{03} observed for three-photon excitation at 885 nm is higher than observed with 702 nm excitation (0.546, Table 3), which is known to be a two-photon process (Szmacinski et al., 1993). Similar values of r_{0i} and the rotational correlation times were found for the Ca^{2+}-bound form of Indo-1. The larger value of r_{03} provides strong evidence for three-photon excitation, which for colinear transitions is expected to result in a more highly oriented excited-state population. The extent to which the excitation follows $\cos^2\theta$ or $\cos^6\theta$ photoselection can be estimated from the ratio of the anisotropies for one-photon and three-photon excitation. For colinear transitions the expected ratio is r_{03}/r_{01} = 1.667. The observed ratio observed for Indo-1 is 1.702. These results suggest that the anisotropy values of Indo-1 are closely approximated by $\cos^2\theta$ and $\cos^6\theta$ photoselection for one- and three-photon excitation, respectively.

The rotational correlation times that were found to be essentially independent of excitation wavelength are independent of the presence or absence of Ca^{2+} (Table 3). Additional evidence of the similarity of the correlation times is seen from the global analysis with variable r_{0i} values of each excitation wavelength and the same global correlation time for all wavelengths. The global fit yields a moderately low value of χ_R^2, with the same r_{0i} values and a single value of θ_r (Table 3). Similar values of r_{0i} were recovered from the global analysis and from the analysis of

each wavelength (Table 3). The similar rotational correlation time observed for each excitation wavelength indicates that the samples were not being significantly heated by the relatively strong 885-nm illumination.

We also measured the steady-state anisotropies of Indo-1 at the wavelengths available from the Ti:sapphire and dye lasers (Fig. 5). One may notice that the one-photon anisotropy of Indo-1 is higher for excitation near 350 nm than for 300-nm excitation, which suggests a single electronic transition for excitation at 351 nm, and overlapping transitions for 295 nm excitation. The steady-state anisotropy becomes progressively higher for excitation at 351, 570–800, and 880–920 nm. The anisotropies for excitation at 351, 570–800, and 840–920 nm are consistent with single colinear absorption and emission transitions moments, for one-, two-, and three-photon excitation, respectively. These steady-state anisotropies are in excellent agreement with that expected for a fluorophore with a 350-ps correlation time and the r_{0i} values listed in Table 3. The r_{0i} values and steady-state anisotropies do not appear to be altered by binding of Ca^{2+} (Table 3).

Calcium-sensitive emission spectra with one-, two-, and three-photon excitation

Calcium-dependent emission spectra of Indo-1 are shown in Fig. 6 for one-, two-, and three-photon excitation at 295, 590, and 885 nm, respectively. These wavelengths were selected to be consistent with one-, two-, and three-photon excitation of Indo-1 when the one-photon wavelength is 295 nm. The poorer quality of the spectra for 590-nm excitation is due to our use of a picosecond dye laser as the excitation source, whereas the femtosecond Ti:sapphire was used for 885-nm excitation. Because the signal intensity depends on the square or cube of the peak laser power, one expects significantly smaller signals using picosecond rather than femtosecond pulses.

TABLE 3 Anisotropy decay and steady-state anisotropies of Indo-1*

Exc. (nm)	No Ca^{2+}				40 μM Ca^{2+}			
	θ_R (ns)	r_{0i}	χ_R^2[‡]	r[¶]	θ_R (ns)	r_{0i}	χ_R^2[‡]	r[§]
295	0.29	0.176	1.4	0.06	0.37	0.262	2.6	0.07
351	0.39	0.372	4.5	0.083	0.37	0.370	5.11	0.085
702	0.33	0.546	4.3	0.112	0.39	0.504	2.0	0.109
885[c]	0.37	0.633	4.1	0.129	0.36	0.626	5.0	0.127
Global analysis[‖] No Ca^{2+} (θ_R = 0.35 ns; χ_R^2[‡] = 6.8)					Global analysis 40 μM Ca^{2+} (θ_R = 0.38 ns; χ_R^2 = 4.3)			
295	—	0.158	—	—	—	0.260	—	—
351	—	0.390	—	—	—	0.367	—	—
702	—	0.532	—	—	—	0.514	—	—
885[c]	—	0.634	—	—	—	0.606	—	—

*Emissions were observed using three 4–96 Corning filters (350–600 nm).

[‡]Uncertainty for phase angles were $\delta\phi$ = 0.4 degrees and for modulations δm = 0.009 (used in Eq. 6) (Lakowicz et al., 1993).

[§]The calcium concentration was 250 μM.

[¶]Steady-state anisotropy measured at each excitation wavelength.

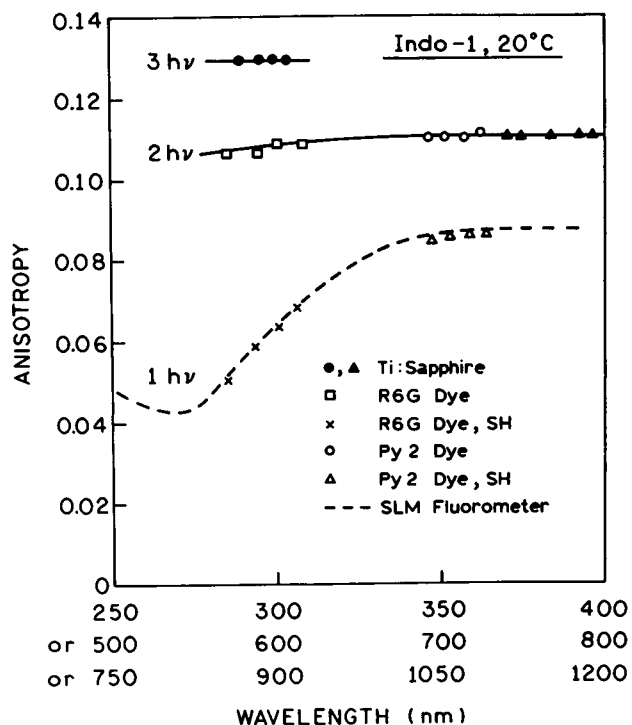[‖]The correlation time is a global parameter, and the amplitudes are nonglobal.

FIGURE 5 Steady-state anisotropy spectra of Indo-1 in the absence of Ca^{2+} for one-, two- and three-photon excitation. SH, second harmonic of the dye laser. The top, middle, and bottom wavelength numbers are for one-, two-, and three-photon excitation, respectively.

For 295-nm excitation the emission is dominated by the Ca^{2+}-bound form of Indo-1. For 885-nm excitation, there is much less emission from the Ca^{2+}-bound form for comparable concentrations of Ca^{2+}. To obtain emission from only the Ca^{2+}-bound form of Indo-1 for 885-nm excitation we used a higher Ca^{2+} concentration of 250 μM. This result suggests that the relative cross section for three-photon excitation of Indo-1 is less for the Ca^{2+}-bound form, as compared to the cross section for one-photon excitation. Of course, the three-photon cross sections are likely to depend on excitation wavelength, so that one expects the wavelength-ratiometric calibration to be different for different modes of excitation. This point is illustrated by the emission spectra observed for 590-nm excitation, which are intermediate between the one-photon- and three-photon-induced emission spectra. Importantly, the emission spectra for 885-nm excitation rule out excitation of Indo-1 by the third harmonic at 295 nm. If this occurred, then the spectra would be identical to those observed at 295-nm excitation. The observation of different relative intensities for a mixture of fluorescent species for one- and three-photon excitation can be regarded as evidence against third harmonic generation in the sample and one-photon excitation by this harmonic (Cable and Albrecht, 1986).
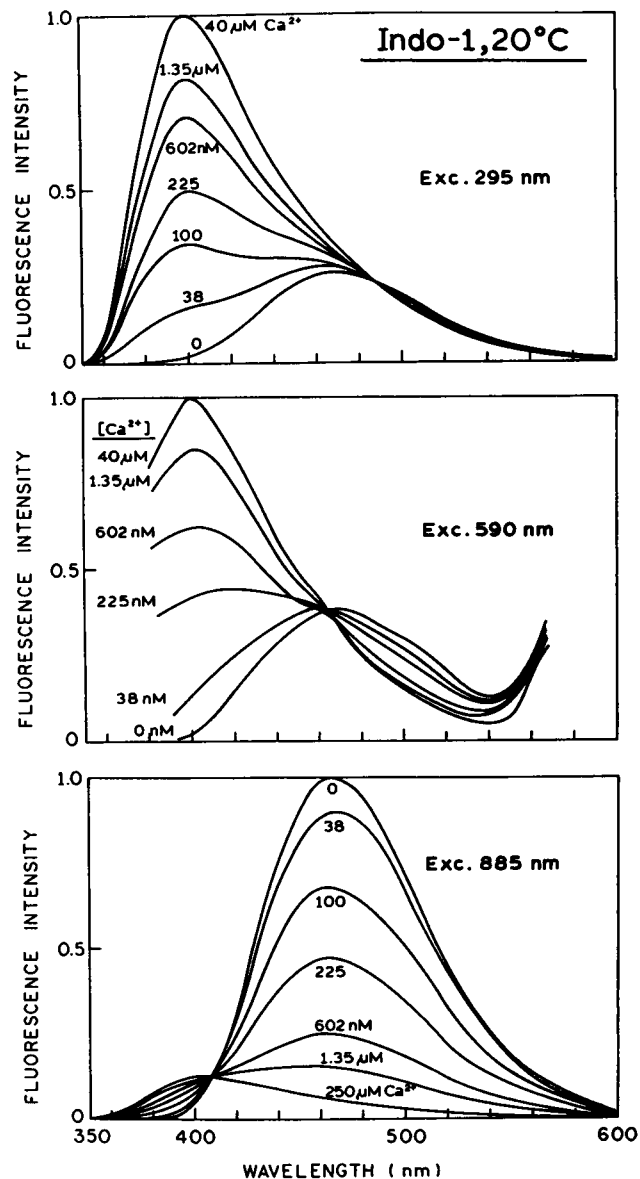

FIGURE 6 Calcium-dependent emission spectra of Indo-1 for one-, two-, and three-photon excitation, at 295, 590, and 885 nm, respectively.

And finally, we examined the intensity-ratio calibration curves of Indo-1, for one-, two-, and three-photon excitation (Fig. 7). In these data the Ca^{2+} dissociation constant can be seen from the midpoint of the ratio curves because of our use of an iso-emissive wavelength for each curve. The same Ca^{2+} dissociation constants were obtained for each mode of excitation, 250 $\pm$ 25 nM. This result indicates that Indo-1 can be used as an intensity ratiometric probe with one-, two-, and three-photon excitation.

It should be noted that all the measurements of Indo-1 with three-photon excitation were performed with a moderately low Indo-1 concentration of 15 μM. This concentration is comparable to that used in fluorescence microscopy, suggesting that three-photon excitation can be of practical importance even in the demanding application of intracellular calcium imaging.
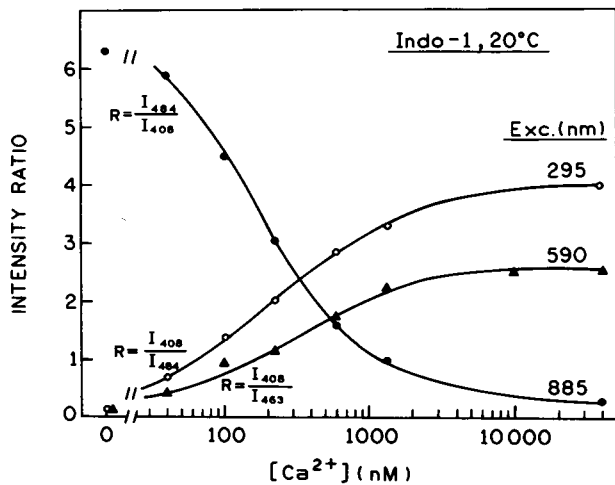
FIGURE 7   Calcium-dependent emission intensity ratios of Indo-1 for one-, two-, and three-photon excitation, at 295, 590, and 885 nm, respectively.

DISCUSSION

Three-photon excitation has a number of potential advantages in time-resolved fluorescence and for fluorescence microscopy. The fundamental output of Ti:sapphire lasers is most intense from 800 to 900 nm, which is suitable for three-photon excitation of UV-absorbing fluorophores potentially including the intrinsic fluorescence of proteins. The Ti:sapphire fundamental wavelengths are above the absorption bands of most biological chromophores, suggesting that the amounts of autofluorescence will be minimal for three-photon excitation. Furthermore, these wavelengths are below the absorption bands of water, suggesting that the biological samples will not be excessively heated by 800–900-nm illumination. An additional advantage of these excitation wavelengths is that they are poorly detected by most photomultiplier tubes (PMTs), and PMTs without significant sensitivity above 600 nm are readily available. Hence experimental problems due to stray or scattered excitation should be easily minimized with three-photon excitation.

A further advantage of three-photon excitation is the smaller excited volumes. In the case of two-photon excitation the absence of signal from outside the focal region has been used to avoid out-of-focus signal (Denk et al., 1990; Webb, 1990; Hell et al., 1994; Stelzer et al., 1994; Piston et al., 1992; Denk, 1994; Denk et al., 1994). By visual comparison of the emission from cuvettes containing fluorophores that display two- or three-photon excitation, we have observed that the emission is seen from a smaller area for three-photon excitation. This indicates that higher spatial resolution in laser scanning microscopy can be obtained by three-photon excitation. One can also predict improved contrast in pattern photobleaching with evanescent wave illumination, as has already been calculated for two-photon excitation (Huang and Thompson, 1993).

In recent publications we have shown that under conditions of two-photon excitation the emission can be simul-taneously quenched by the excitation pulse (Gryczyński et al., 1993; Gryczynski and Lakowicz, 1994). Light quenching depends on overlaps of the emission spectrum of the fluorophore with the excitation wavelength (Lakowicz et al., 1994a), which is a common occurrence with two-photon excitation. However, with three-photon excitation the wavelengths will generally be at wavelengths longer than the emission spectra, so that the intense pulses are not likely to cause light quenching. The use of three-photon excitation may prevent light quenching in laser scanning confocal microscopy with two-photon excitation, where the intensely focused excitation can overlap with the emission spectra of the fluorophores.

A final advantage of three-photon excitation can be selectivity in the excitation process. In an initial survey of ten fluorophores we noticed a wide range of three-photon-induced signals, and only a few of these fluorophores displayed significant three-photon-induced emission. In fact, Indo-1 appeared to be unique in displaying a high cross section for three-photon excitation. This suggests that only a limited number of species will display three-photon excitation, thereby allowing selective excitation in the presence of other fluorophores.

In closing we note that the reader may regard simultaneous absorption of three photons to be an exotic process with little practical use. However, the same could have been said about two-photon excitation, which can now be regarded as a promising research tool in biophysics and cell biology.

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